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(54) Title: BIOCONTROL AGENTS FOR USE IN AGRICULTURE

(57) Abstract

A biocontrol agent which includes or is derived from a sourdough starter formulation comprising a mixed culture of the yeast component, a bacterial component and a substrate for the mixed culture. The agent can be used for reducing or alleviating fungal infection of plants or fruit.

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TITLE

"BIOCONTROL AGENTS FOR USE IN AGRICULTURE"

FIELD OF THE INVENTION

THIS INVENTION relates to biocontrol agents for use in agriculture.

PRIOR ART

Conventional biocontrol agents for use in horticulture are summarised in an article by J. M. Whipps entitled "Status of Biological Disease Control in Horticulture" Biocontrol Sci & Technol (1992) 2 3-24. In this article emphasis is placed on research relating to the use of biological disease control agents in horticulture which may include vegetables, fruit, ornamentals, flowers and protected crops.

In this article it is established that the three main direct biocontrol mechanisms are:-

(i) parasitism or predation of one organism by another;

(ii) antibiosis, where antagonists secrete metabolites harmful to plant pathogens; and

(iii) competition where demand exceeds immediate supply of nutrients or space.

These actions can take place outside or within the plant. Other mechanisms include production of cross protection or induced resistance involving inoculation of a plant with a micro organism which is non pathogenic or only mildiy pathogenic which results in the plant becoming resistant to subsequent challenge. Also inoculation of plants with pathogens of reduced pathogenicity carrying double stranded RNA or DNA plasmids may result in decreased virulence in the overall population of pathogens.

However, it is also pointed out in the Whipps article that until the modes of action can be shown to occur in soil or on or within plants growing in natural environments, the significance of any of the abovementioned mechanisms must be viewed with reservation.

It is also noted that a major problem facing all biological control agents is that they are generally expected to match the efficacy of existing

chemical methods under all conditions as described in Powell & Faull Biotechnology of Fungi for Improving Plant Growth, Cambridge University Press (1989) p 259-275. This is pointed out in the Whipps article as being an objective which may not always be possible. An example of this is that the existence of specific suppressive soils illustrates the influence that soil conditions can have in biocontrol. Similarly inoculum potential of the pathogen is known to be important to the degree of biocontrol achieved but is often ignored as described in McQuilken *et al.*, Plant Pathology 39 452-462 (1990) and Budge and Whipps Plant Pathology 40 59-66 (1991).

Direct application of biological control agents to aerial, root and soil microbiomes before, during and after plant growth has also been attempted as described in the Whipps article but relatively few of these techniques have been shown to be commercial in use.

In summary therefore, biocontrol techniques in horticulture have not been commercially successful and one reason for this is that there is often a lack of reproduceability between trials *in vitro* and in the field. Also when compared to chemicals biological techniques suffer because of inoculum production, application and cost. Chemicals are also often preferred on the grounds of efficacy because chemicals generally work irrespective of environment or inoculum potential. Chemicals also are easier to apply to target plants and also a greater range of pathogens may be controlled.

However, the use of chemicals such as fungicides e.g. in control of storage rot of fruit and vegetables which may be caused by specific plant pathogens is now becoming unpopular because they are hazardous to the health of humans and are also considered to be detrimental to the environment. This is specifically discussed in Agricultural Research, April 1990. This reference describes a strain of yeast that may be used to control fruit rot. However, again commercialisation of such biocontrol agents is difficult because of the time, effort and expense involved not only in finding biocontrol agents but also verifying their effectiveness.

In Neth. J. Pl. Path 91 (1985) p 265-267 by Williamson *et al.*, there is described the use of as biocontrol agents the yeasts *Sporobolomyces roseus*

and *Cryptococcus laurentii var flavescens* in relation to control of the pathogen *Colletotrichum graminicola* in maize plants. It was found that the yeasts reduced lesion density and necrosis from *C. graminicola* by approximately 50%. This reference also made the observation that naturally occurring yeast populations may have a moderating effect of maize anthracnose, especially in conjunction with selected fungi. However, the same problems described above in relation to biocontrol agents also apply to this reference.

Reference also may be made to U.S. Patent 5,041,384 which described various strains of *Pichia guilliermondi* which were isolated from the surface of citrus fruits and which are useful in controlling a variety of fruit rot pathogens in a variety of fruits.

Other references which describe the use of yeasts or fungi as well as bacteria obtained from a natural source include (i) EP 485440 which describes a new yeast strain obtained from the surface of citrus fruits and which may be used to control fruit rot pathogens; (ii) U.S. Patents 5,047,239 and 4,764,371 which describes the use of a strain of *Bacillus subtilus* for biological control of fruit rot; (iii) U.S. Specification 4,377,571 which describes the use of *Pseudomonas syringae* for treatment of Dutch elm disease; (iv) U.S. Specification 4,950,472 which describes the use of a new strain of *Acremonium breve* in controlling grey mould infection of pome fruit; and (v) U.S. Specification 4,975,277 which describes an isolate of *Pseudomonas cepacia* for biological control of post harvest disease in fruit.

In Japanese Patent JP 3077803 reference is made to *Pseudomonas* bacteria selected from *P cepacia, P gladioli, P picketti, P vorans, P dimunata* and *Bacillus* bacteria selected from *B cereus, B mycoides, B anthracis* and *B thuringiensis* as biocontrol agents in relation to soil borne diseases.

U.S. Patent 4,878,936 describes the use of *B cereus* ATCC 53522 as having biocontrol activity and which produces a fungicide active against *Phytophera megasperma*.

In Russian Patent SU 237480 reference is made to a strain of *B cereus* useful as a biocontrol agent for protecting plants against insects.

In U.S. Patent 4,661,351 reference is made to compositions

containing biosynthetic pesticidal products obtained from *Bacillus* bacteria selected for *B thuringiensis*, *B sphaericus*, *B popilliae*, *B cereus*, *B lentimorbus* or *B friboungensis*. These bacteria may also be provided with a low melting point polyester as described in Patent EP 145087.

In Patent Specification JP 59082085 there is described a method of controlling harmful insects by use of a biocontrol agent containing *Bacillus subtilus*, *Bacillus coagulans*, *Micrococcus luteus*, *Bacillus stearothermophilus*, *Clostridium pasteurianum*, *Clostridium aminovalericum*, *Clostridum thermosaccharolyticum* and *Thermoactinomyces vulgaris*.

Reference also may be made to a publication by Broadbent et al entitled "Bacteria and Actinomycetes Antagonistic to Fungal Root Pathogens in Australian Soils" which includes *B subtilus*, *B megaterium*, *Streptomyces spp*, *B cereus*, *B pumilus*, *B polynyxa*, *B badius*, *Pseudomonas putida*, *P fluorescens* and *Pseudomonas spp* as having biocontrol activity.

Reference may also be made to Visser et al Applied and Environmental Microbiology 52 552-555 (1986) which describes a variety of lactic acid bacteria isolated from plant surfaces and plant associated products which were found to be antagonistic to the phytopathogens *Xanthomonas campestris*, *Erwinia catotovora* and *Pseudomonas syringae*. In pot trials, treatment of bean plants with a *Lactobacillus planetarium* strain before inoculation with *Pseudomonas syringae* caused a significant reduction of the incidence of disease.

From a review of the abovementioned prior art, it is clear that it is well known to take natural isolates which may be found on leaf surfaces of fruit and cultivate such natural isolates for their use as biocontrol agents when applied to crops or fruit and other plants. The natural isolates may be removed from the leaf surfaces and cultivated in suitable media. In some cases culture media may be dried and ground into a powder before being incorporated in a carrier. In some cases the culture media may be mixed with wax such as a water or paraffin/mineral oil base. Sometimes the infected fruit may be dipped into a solution containing the natural isolate.

However, it would seem that one important disadvantage of the use

of natural isolates is that this is often approached in a random manner and that the relevant antagonist must be identified and isolated before being used in field trials. There is also no evidence that the use of natural isolates has been adopted on a commercial scale.

It would also seem clear that the main disadvantage of use of biocontrol agents such as *Bacillus thuringiensis* is the cost because of their need to be applied more often than chemicals and also because of loss of potency within a relatively short time.

It is also clear when use is made of genetic engineering techniques to produce biocontrol agents such as recombinant strains or proteins obtained from recombinant strains that such techniques are expensive and also cannot be sold on the market until obtaining approval from the regulatory authorities such as the U.S. Food and Drug Administration. Also extensive field trials are required before commercial evaluation can be considered.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a biocontrol agent which may alleviate the abovementioned problems of the prior art.

The biocontrol agent of the invention may include or be derived from:

(i) a mixed culture of a yeast component and a bacterial component; and

(ii) a substrate for the mixed culture (i).

The yeast component is suitably useful for producing a leavening or rising action in a sourdough which are doughs in which flour (for example, rye flour) is fermented by lactic acid bacteria rather than by bakers yeast. Sourdoughs are described, for example, by Oura *et al.* in Economic Microbiology, Volume 7 entitled "Fermented Foods" edited by A. H. Rose pages 123-134. Such yeasts may be selected from *Candida krusei*, *Saccharomyces cerevisiae*, *Saccharomyces exiguss* and *Pichia saitoi*. However, any yeast could be utilised which is effective in combination with an appropriate bacterial component and a suitable substrate to produce as main

products lactic acid, acetic acid, ethanol and carbon dioxide which is the hallmark of sourdough fermentation. In sourdough fermentation a glycolytic pathway is characterised by homofermentative lactic acid bacteria via aldolase and a heterofermentative lactic acid bacteria pathway for glucoses and pentoses via phosphoketolase.

Saccharomyces exiguss is alternatively known as *Torulopsis holmii*. Other yeasts that may be used include yeasts of the genus *Saccharomyces* generally which include as mentioned above *S. cerevisiae*, *S. exiguss*, *S. inusitatus* and *S. uvarum*.

The bacterial component of the symbiotic mixed culture may be selected from *Lactobacilli* as well as members of the genera *Leuconostoc*, *Pediococcus* and *Streptococcus* of the family *Streptococcaceae*. Different species of Lactobacilli may be used depending on th eir ability to use different substrates. Typical lactic acid bacteria may be selected from *L. plantarum*, *L. casei*, *L. delbreuckii*, *L. leichmannii*, *L. brevis* (especially var lindneri), *L. fermentum*, *L. pastorianus*, *L. bucheri*, *L. acidophilus*, *L. farciminis*, *L. alimentarius*, *L. fructivorans*, *L. viridescens*, *L. cellobiosus* and *L. solivarius*. Other bacteria that may be used include *Pediococcus cerevisiae*, *P. acidilactici* as well as *Citrobacter* spp or *Micrococcus* spp.

An especially preferred bacteria for use in the biocontrol agent of the invention are *Lactobacillus sanfrancisco* which are used in the San Francisco sourdough "French bread" process. These bacteria are indifferent to oxygen and do not use carbohydrates other than maltose as a carbon source. *L. sanfrancisco* form a symbiotic relationship with *Saccharomyces exiguss* which mainly performs the leavening function.

Further information on sourdough cultures may be obtained from:

(i) Nout Int. J of Food Microbiology 12 217-224 (1991);

(ii) Nout *et al.*, J of Applied Bacteriology Symposium Supplement 1992, 73 1365-1475;

(iii) Saunders *et al.*, Cereal Chemistry 49 86-91 (1972);

(iv) Cooke *et al.*, FEMS Microbiology Reviews 369-379 (1987);

(v) Sriraanganthan *et al.*, Applied Microbiology 25 461-470 (1973);

(vi) Kline and Sugihara Applied Microbiology 21 459-465 (1971); and

(vii) Ng Applied and Environmental Microbiology 31 395-398 (1976).

In a specific form of the biocontrol agent of the invention, which has been deposited at the Australian Government Analytical Laboratories as discussed in more detail hereinafter (herein referred to as SDBS), the Lactobacilli which were located in the deposited mixed culture included (i) *L. parabuchneri* which was characterized by Phillips and Collins in 1988, (ii) *L. parabuchneri/brevis* strain, and (iii) *L. casei ssp casei* which was characterized by Rogose *et al.* in 1953. However, differentiation between *L. buchneri* and *L. parabuchneri* can be very difficult and it is within the scope of the invention to provide a mixed starter culture which includes *L. buchneri* in substitution of *L. parabuchneri* and other strains of *L. casei* for example in substitution of *L. casei ssp casei*. Differentiation may also be extremely difficult between *L. parabuchneri*, *L. buchneri* and *L. brevis* and *L. brevis* may also be substituted for *L. parabuchneri* or *L. buchneri*.

A sample of the mixed culture was forwarded to Kluyver Laboratory of Technology Delft Technical University in The Netherlands and Lactobacilli strains (i), (ii) and (iii) were identified. The yeast component of the mixed culture was also determined as *Saccharomyces cerevisiae* Meyen ex Hansen by CBS Yeast Identification Service Delft, The Netherlands.

The substrate utilised in the mixed culture may comprise any preparation derived from cereals such as flour and in particular rye flour or white flour which may include unbleached white wheat flour. Molasses may also be utilised as a substrate.

The substrate may also be mixed with water which is suitably "pure water" comprising bottled rainwater or sterilised Milli-Q filtered water.

The pH of the mixed starter is suitably in the range 3.0-6.0 and more suitably 3.0-4.5.

Fermentation may be initiated by mixing equal parts flour and water to form a paste, and this is then added at a suitable ratio (e.g. 1:1) to starter

culture. Incubation may occur over 5-48 hours but more suitably 24 hours.

Advantages of the bicontrol agent of the invention over conventional pesticides and biocontrol agents include the following:

(i) ease of registration as a fungicide because components of the mixed culture have been utilised in food production for a relatively long period;

(ii) the bacterial component such as the lactobacilli are claimed to have probiotic effects on the organisms which consume them. This means that health is enhanced by improving the balance of bacteria and yeasts or microflora of the gut;

(iii) the biocontrol product of the invention is compatible with sustainable agriculture and free from health risks which is not the case with conventional chemical or antibiotic fungicides as discussed above which are disadvantageous because of:

(a) high oncogenic risks;

(b) loss of utility because of the development of fungicide resistant strains of many pathogens; and

(c) pressure from environmental groups for lessening dependence on toxic chemicals because of dangers to the environment and the development of sustainable cropping systems.

The biocontrol agent of the invention may also include other components so as to facilitate its mode of action in a desired application.

Thus one component may include a carrier which may help to establish and maintain antagonistic populations of the biocontrol micro-organism on the target surface. This carrier may be a lipid such as wax or oils inclusive of coconut oil, vegetable oil, olive oil, canola oil or emulsions inclusive of fish oil emulsion. Suspensions or slurries may also be used consistent with the above criteria. One suitable carrier is an emulsified vegetable oil identified by the trade mark CODACIDE. Another lipid carrier is identified by the trade mark NU-FILM 17. The use of the lipid may also enhance dispersability and may also be used as an adhesive to the target

surface.

Another component that may be utilised is a thickener which is suitably a gum such as natural or modified gums inclusive of algin, carrageenan, xanthan and tragacanth gums. A gum may also be used as an emulsifier or stabiliser.

Examples of pathogens of aerial surfaces of crop plants or crop products which may be subjected to the biocontrol agent of the invention include mainly fungal pathogens i.e. *Colletotrichum* (anthracrose), *Cercospora* (spots and blights), *Botrytis* (grey mould and rots), *Alternaria* (leaf spot), Monilinia (brown rot), *Penicillium* (blue mould) and Rhizopus (soft rot).

Other components that may be included in the biocontrol agent of the invention include micro-organism antagonism enhancer(s) which may include MgO, $MgCO_3$ and/or $CaCl_2$, pathogen inhibitors which may include tea polyphenols (especially epicatechin gallate and epigallocatechin gallate) as well as antioxidants and substances to facilitate production, storage and/or application of the agent.

It will also be appreciated that the sourdough biocontrol products of the invention may be formulated for commercial purposes in any suitable manner and thus may be freeze dried to improve storage life.

The biocontrol agent of the invention may be applied to the target before, during and after harvest.

EXPERIMENTAL

FERMENTATION. The SDBS starter culture was stored at 4°C before use. To reactivate, equal parts of "Kialla" organically grown unbleached white flour and Milli-Q filtered water were mixed to form a smooth paste. The paste was then combined with the starter culture (1:1) sometimes it may be necessary to fully decant off the "clear layer" of accumulated acetic acid, lactic acid and ethanol from the top of the starter culture when this is more than ⅓ of the total volume. The culture was then incubated in a loosely covered glass jar at room temperature (23-30°C) for mostly 24 hours but up to five days.

MANGOES. In Experiment 1, naturally infected green fruit were

desapped and then graded into two groups according to the density of lesions, i.e. mild natural infection and severe natural infection. In Experiment 2, the naturally infected fruit were desapped and then graded according to the level of natural infection. Fruit were then distributed to 6 groups, each group consisting of 3 fruit displaying a similar range of infection levels.

TREATMENTS. The treatments applied in Experiments 1 and 2 are summarised in Table 1. In both experiments, fruit were dipped in Milli-Q filtered water (control) or SDBS (Treatment /± additives) for 1 minute and incubated on a self draining rack for 6 (Experiment 2) or 7 (Experiment 1) days in the laboratory at room temperature (23°C). In Experiment 2a, in which fruit were dipped 3 times, dipping was carried out on days 1, 2 and 3 of the experiment. The additives tested in Experiment 2b, Nufilm 17 (Miller Chemical and Fertiliser Corporation USA) and Codacide (Spraytech Australasia Pty. Ltd.) were added to the dipping mixture at the rate and in the manner recommended in the instructions.

DISEASE ASSESSMENT. Anthracnose disease development on the fruit was assessed after 6 days (Experiment 2) or 7 days (Experiment 1) according to the following disease rating scale:

Disease Rating	% Fruit Surface Infected
1	0 - 25
2	26 - 50
3	51 - 75
4	76 - 100

RESULTS. In the first experiment (FIG 1), the amount of anthracnose disease development in both mildly and severely infected groups of mangoes was reduced by one disease rating level by SDBS treatment. Furthermore, on the SDBS treated mangoes, typically lesions were localised and necrotic rather than the sunken and spreading type which predominated on the control fruit.

Repeated dipping on each of the first three days of the experiment (Experiment 2a), reduced the disease rating for the fruit from level 3 to level 1, so that the fruit ripened with only the localised necrotic lesions from the

natural infection.

In Experiment 2b, treatment with one dip of SDBS achieved a single reduction in the amount of anthracnose developing on the mangoes. The addition of the spreader - sticker formulations Nufilm 17 and Codacide reduced disease ratings by 2 levels (FIG 2), to give much the same level of disease control achieved with 3 dips. SDBS with Nufilm 17 achieved the best and most consistent disease control.

When the SDBS mixtures were washed off the treated fruit and the clean fruit incubated for a further three days in the laboratory at room temperature, the anthracnose developed in the same manner observed on control fruits.

The SDBS mixture appears to act fungistatically to inhibit or retard the development of anthracnose on naturally infected common mango fruits. The fungistatic effect of the treatment can be eliminated when the mixture is removed from the fruit surface by washing. The effect can be enhanced by prolonged exposure to the actively growing (wet) mixture by repeated dipping or by the addition of the spreader - sticker formulations Nufilm 17 and Codacide.

The SDBS product has now been deposited at the Australian Government Analytical Laboratories, Suakin Street, Pymble, New South Wales, Australia on March 5, 1993 and has been allocated accession number N93/9578. In 1 g of SDBS starter culture there is 0.37 g flour, 0.63 g water up to 10^{12} cfu/g of lactobacilli and 2 x 10^3 cfu/g *Saccharomyces cerevisiae*.

The SDBS starter culture originated from a sourdough starter which was developed by Mrs Edith Aylward of Roys Road, Palmwoods, Queensland, Australia. In bread making operations carried out by Mrs Aylward, the starter initially comprised a combination of intact grapes of the Cabernet variety and a commercial starter obtained from Germany called "BACK FERMENT" which was utilised in rye bread manufacture and such commercial starter was mixed with water and unbleached organic white flour ("KIALLA") in equal volumes. A fermentation occurred and the fermentation vessel was lightly covered and left standing at RT for up to seven days. After bubbling or frothing took place

the ferment was decanted to remove grapeskins and stalks and was placed in a refrigerator at 4°C in a sealed jar for a resting period of 1-2 days. Subsequently 50% of the preparation was used for bread making where yeast, salt and flour was added and the remainder supplemented with flour and water and retained in the refrigerator at 4°C for the next fermentation. Expansion of the starter by fermentation of organic unbleached white flour with culture from the previous week occurred weekly over a period of five years and also in a totally non-sterile fashion so that the starter may have also included micro-organisms introduced from the flour. Mrs Aylward, from these operations, has produced a sourdough bread which has been unaltered in flavour or texture and smell.

CHARACTERISTICS OF SDBS - RELATIVE DENSITIES OF MICRO-ORGANISMS IN MIXTURE.

It is not possible to distinguish between the different species of lactobacilli of the SDBS starter culture on plates. We always obtain best growth of all the species isolated on MRS so we have used this medium for total lactobacilli counts. *Lactobacillus casei* ssp caesi grows well on Lactose LB medium, while the other isolates either do not grow or only grow very slowly and poorly on this medium. We have used this medium to estimate the proportion of *L. casei* ssp casei in the total count.

The data in Table 2 show that the total number of lactobacilli increase from about 1 million per ml of ferment to a peak of about 7 million per ml after 24 hours incubation. The total bacterial population seems to remain at around this level for the following 24 hours.

The size of the population of *L. casei* measured in these experiments, indicate that it is an important component of this mixture. Unfortunately, we were unable to find a medium suitable for differentiating between *L. parabuchneri* and the *L. parabuchneri/brevis* strain.

As discussed below yeast population growth seems to be very sensitive to both oxygen availability and organic acid and ethanol concentration. If yeast numbers are low in the starter at the beginning of the fermentation (in our experience due to prolonged growth and/or storage under

anaerobic conditions), the population will not grow and very little ethanol production will occur. When reasonable numbers are present $>10^3$ per ml, the population seems to be active in the first 24 hours of fermentation producing ethanol. This can be seen in the fermenting mix as it becomes very frothy. As the fermentation proceeds, acid accumulation and possibly oxygen depletion will limit yeast growth and activity. When this occurs the mixture stops frothing and dies down again.

Yeast numbers show enormous variation between experiments. In Table 2, when we realised that their numbers were very low after Run 2, we altered fermentation conditions to make conditions more aerobic to encourage yeast growth. The steady increase in their numbers over the three runs would suggest favouring aerobic conditions early in fermentation allows them to grow, before their activity is suppressed by increasing acidity and ethanol concentration of the ferment.

Table 3 illustrates media on which the isolates of SDBS will grow as pure cultures. In Table 3, LLA medium comprised Lactic Acid Bacteria Media using lactate. This medium comprised 0.03% Tween 80, 1.5% fresh yeast extract, 0.6% tryptone and 2.0% lactose which was adjusted to pH 5.45 with agar. SLA medium is the same as LLA medium except sucrose is utlised instead of lactose at the same concentration. MLS medium is the same as LLA medium except maltose is used instead of lactose at the same concentration.

RSYM medium comprised 10% raw sugar, 0.05% $(NH_4)_2HPO_4$ 1.5% fresh yeast extract and 2% agar. Water is added to 100% and the pH adjusted with 1N HCl to pH 6. The medium should be autoclaved before use. SYM medium is the same as RSYM with sucrose being used instead of raw sugar. MEA is a conventional malt extract medium obtained from Oxoid Australia. PDA is a conventional potato dextrose medium also obtainable from Oxoid Australia. RAA is a conventional rogosa agar medium obtainable from Oxoid Australia. Molasses media compriess 1%, 2%, 5%, 10% or 20% molasses in water which is autoclaved before use. MRS medium is also obtainable from Oxoid Australia where 52 g of MRS broth is dissolved in 1000

ml of MilliQ H_2 O and 20 g of agar is added. The mixture is autoclaved at 121°C for 15 minutes.

During the fermentations shown in Table 4, although total *L. casei* and yeast numbers end up returning to approximately the same levels in each of the four mixtures after 48 hours incubation, total lactobacilli numbers do not seem to recover as well.

In the standard fermentation, 50% of the mixture is the starter culture. When we carried out fermentations in which the starter culture component was reduced to 20%, 10% and 5% as shown in Table 5, we found that after 24 hours fermentation normal levels of ethanol, lactic and acetic acid were produced. Although pH tended to be slightly higher after 24 hours, pH was normal in all the fermentations after 24 hours.

Table 6 shows pH profiles of the standard fermenation at 30°C.

All data represent means of measurements made in triplicate from two or three replicate fermentations. Ethanol measurements made using Boehringer Mannheim test kit, Acetic and Lactic acids Boehringer Mannheim test kits and HPLC (Biorad Aminex HPX-87H column).

In Table 6, the freshly prepared mixed culture has a pH around 3.8 becoming slightly more acid over time with a 48 hour pH around 3.4 Lowest pH recorded with wheat flour substrate was pH 3.2.

In Table 7, which shows typical profiles of ethanol production, the fermentation starts with a residual level of ethanol in the mix from the starter at around 2 g/L. Production peaks after 24 hours at around 7 g/L, then tapers off as acid levels increase.

Ethanol production relates directly to yeast density in the mix. In Runs I and 2 yeast numbers were very low at the beginning of fermentation.

Fermentation in Table 8 starts with about 1 g/l acetic acid residual from the starter, which appears to be metabolized as levels drop considerably after 17 hours then slowly increases to reach original levels after 48 hours.

Residual levels lactic acid in the starter in Table 9 are relatively high (around 3.5 g/L) and rise steadily during fermentation to reach levels double the 0 time concentrations after 48 hours.

After fermentation, it is noted that mucilage is produced as an end product.

In a standard protocol for carrying out the fermentations shown in Tables 7-10, 200 ml of the standard mixture (1 part starter culture: 1 part flour/water paste) was prepared in triplicate in 250 ml pyrex bottles and incubated for 48 hours in the dark at 30°C. The bottles were loosely covered with aluminium foil to allow aerobic fermentation.

After 0, 17, 24 and 48 hours, the pH of each ferment was measured with a digital stick pH meter (Hanna Instruments). Ethanol and organic acid profiles were described using measurements obtained from the following UV method determination kits, according to the manufacturers instructions:

1. Ethanol - Boehringer Mannheim Ethanol test kit (No. 176290);
2. Acetic Acid - Boehringer Mannheim Acetic Acid test kit (No. 148261);
3. Lactic Acid - Boehringer Mannheim test kit (No. 1112821).

Acetic and lactic acid concentrations were confirmed with a Waters/Applied Biosystems HPLC using a Biorad Aminex HPX-87H column according to the manufacturers instructions.

In relation to the fermentations shown in Tables 4-5, these were carried out exactly as described above except that the amount of starter in the mixture was varied i.e. starter contributed to 5, 10, 20 or 50% (50% = standard mixture) of the total volume of the mixture.

In relation to the fermentations shown in Tables 2 and 4, densities were initially carrying out serial dilutions of each fermentation sample were prepared in sterile water. Viable counts of microflora were made at 160,000 dilution. Yeast numbers were estimated by plating out 100 microlitres of diluted sample onto DRBC plates (DRBC medium is obtainable from Oxoid Australia and has 0.1 g/l chloromphenicol added thereto). Total lactobacilli were counted by plating out 50 microlitres of diluted sample onto MRS medium. *Lactobacillus casei* numbers were estimated by plating out 50 microlitres of diluted sample onto Lactose LB medium.

Plates were incubated in the dark at 30°C for approximately five

days.

Each plating was performed in triplicate and the data represent the mean numbers of each bug calculated from the numbers growing on the three plates. Data are expressed as CFU/ml of ferment.

In regard to Tables 10 and 11, such Tables illustrate experiments which were conducted using a protocol similar to that described in regard to Table 1 which measures in Table 10 the mean diameter of lessions in cm on mango fruit naturally infected with anthracrose over 17 days post treatment with the biocontrol agent of the invention which was prepared as set out in the protocol corresponding to Table 1 above. Table 11 illustrates the increase in density of lesions on naturally infected mango fruit over a 17 day period after treatment.

Table 12 refers to reductions in anthracnose development in mangoes and avocado after post harvest treatment with SDBS.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1 refers to the development of anthracnose on naturally infected mangoes after 7 days incubation at RT as discussed above in relation to Table 1;

FIG 2 refers graphically to the effect of sticker-spreaders on the development of anthracnose on naturally infected common mangoes;

FIG 3 is a flow diagram illustrating a proposed method of manufacture of the biocontrol agents of the invention on a commercial scale.

FIG 4 is a graph illustrating in graphical form the data set forth in Tables 11-12.

FIG. 5 is a graph illustrating in graphical form the data set forth in Tables 6-9.

It will be appreciated from the foregoing that the inventive concept refers to the realisation that sourdough starter formulations surprisingly have a biocontrol capability especially in relation to fungal diseases of plants. This is exemplified by application of the biocontrol agent of the invention to mangoes and avocado which were infected with *Colletotrichum gloeosporioides* and *Collectotrichum acutatum*.

In another aspect of the invention there is provided a process for production of a biocontrol agent for use in biocontrol of fungal diseases in fruit or plants including the steps of:

(i) obtaining a sourdough starter culture including a yeast component and a bacterial component;

(ii) adding said starter culture to a fermentation substrate to form a fermentation broth;

(iii) incubating said fermentation broth for a period of time which is desirably 12-48 hours; and

(iv) forming a liquid from said fermentation broth into a biocontrol formulation and optionally adding a suitable carrier which may include or is selected from a spreader or sticker, dispersant, thickener, emulsifier or stabiliser.

TABLE 1. Summary of the experimental design of Experiments 1 and 2 (a & b)

TREATMENT	NUMBER OF FRUIT	
EXPERIMENT 1	Mild Infection	Severe Infection
Control (water dip)	5	5
SDBS treatment (dip)	5	5
EXPERIMENT 2		
(a)	Z	
Control (3 dips in water)	3	
SDBS treatment (3 dips)	3	
(b)	Z	
Control (1 water dip)	3	
SDBS treatment (1 dip)	3	
SDBS + Nufilm 17 (1 dip)	3	
SDBS + Codacide (1 dip)	3	

TABLE 2. Densities of Lactobacilli and yeasts in the mix.

Group	Run No	CFU/ml over Time (hours)			
		0	17	24	48
Total Lactobacillii ($\times 10^6$)	1	1.23	4.58	4.70	5.44
	2	1.74	5.34	9.12	5.01
	3	1.54	-	6.26	8.60
L. casei ($\times 10^4$)	1	2.78	3.03	2.32	12.30
	2	2.75	2.92	1.92	5.20
	3	2.64	-	2.83	7.36
Total Yeast ($\times 10^3$)	1	$<10^2$	$<10^2$	$<10^2$	$<10^2$
	2	1.13	0.58	1.05	0.47
	3	312.00	-	35.20	67.20

TABLE 3. Media on which the isolates of SDBS will grow as pure cultures.

IDENTITY	BEST GROWTH	SUBOPTIMAL (slow & poor)
Saccharomyces cerevisiae	MEA PDA RSYM SYM %-% Molasses	
Lactobacillus parabuchneri	MRS	LLA MLA SLA RAA
Lactobacillus parabuchneri	MRS	LLA MLA
Lactobacillus parabuchneri	MRS SLA	MLA LLA
Lactobacillus parabuchneri/brevis	MRS MLA	SLA
Lactobacillus casei ssp casei	LLA MRS	MLA SLA
Lactobacillus casei ssp casei	LLA MRS	MLA SLA

TABLE 4. Densities of the micro-organisms in the minimal starter culture fermentation.

Group	% Starter in mix	CFU (x 10^5) per ml starter over Time (hours)		
		0	24	48
Total Lactobacilli	50	15.35	62.60	86.00
	20	2.84	7.36	11.20
	10	2.62	6.10	9.02
	5	1.65	4.10	8.98
L. casei	50	0.26	2.83	0.74
	20	2.70	1.97	0.98
	10	2.16	1.70	0.91
	5	1.65	1.89	1.44
Total Yeasts	50	3.12	0.35	0.67
	20	1.52	0.13	0.45
	10	0.98	0.16	0.37
	5	0.26	0.24	0.43

TABLE 5. Fermentation characteristics using 50%, 20%, 10% and 5% starter culture.

Parameter	% Starter in mix	Time (hours)		
		0	24	48
pH	50	3.22	3.71	3.53
	20	3.88	3.65	3.30
	10	4.45	3.51	3.27
	5	5.04	3.60	3.27
Ethanol (g/L)	50	2.29	3.03	1.56
	20	1.33	3.05	2.96
	10	0.64	3.02	2.84
	5	0.15	3.02	1.94
Acetic Acid (g/L)	50	-	0.96	3.21
	20	-	1.13	2.87
	10	-	1.25	2.54
	5	-	0.89	2.81
Lactic Acid (g/L)	50	2.74	7.46	4.95
	20	1.52	8.07	9.21
	10	0.70	7.41	9.01
	5	0.49	7.95	6.37

TABLE 6. Typical pH profile

Run No	Time (hours)			
	0	17	24	48
1	3.87	-	3.43	3.40
2	3.73	3.45	3.37	3.29
3	3.73	3.45	3.44	3.43
Mean	3.78	3.45	3.41	3.37

TABLE 7. Typical profiles of Ethanol production (g/L)

Run No	Time (hours)			
	0	17	24	48
1	-	1.34	2.40	1.50
2	0.87	1.36	2.60	1.00
3	2.31	2.90	7.06	5.74
4	2.29	-	3.03	1.56

TABLE 8. Typical profiles of Acetic Acid production (g/L)

Run No	Time (hours)			
	0	17	24	48
1	-	1.92	2.69	2.80
2	0.76	0.39	0.65	1.29
3	0.99	0.28	0.44	1.08
4	-	-	0.96	3.21

TABLE 9. Typical profile of Lactic Acid production (g/L)

Run No	Time (hours)			
	0	17	24	48
1	-	-	-	-
2	4.69	7.36	8.39	9.45
3	3.30	6.15	5.90	7.45
4	2.74	-	7.46	4.95

TABLE 10. Mean diameter of lesions (cm) on Mango fruit naturally infected with anthracnose over 17 days post treament (Mean 20 lesions per 16 fruit per treatment)

Days after treatment	Treatments			
	Control (water dip)	SDBS (1 dip)	SDBS (3 dips)	SDBS + Yeast (1 dip)
2	1.48	1.39	0.70	0.32
4	3.15	1.68	1.15	0.76
7	8.66	2.40	2.56	2.73
9	11.35	4.12	4.65	4.50
11	25.39	6.94	7.46	6.25
14	36.40	18.32	23.3	15.4
17	44.15	27.02	24.4	24.2

TABLE 11. Increase in density of lesions on naturally infected Mango fruit over 17 day period after treatment (Mean no. per 6, 4 cm^2 quadrates per fruit, 16 fruit per treatment)

Days after treatment	Treatments			
	Control (water dip)	SDBS (1 dip)	SDBS (3 dips)	SDBS + Yeast (1 dip)
2	0.19	0.20	0.16	0.01
4	0.85	0.20	0.19	0.19
7	2.33	0.52	0.23	0.77
9	2.70	1.4	1.03	1.65
11	3.74	2.86	1.85	2.37
14	5.29	3.52	3.46	3.38
17	6.32	6.01	4.71	6.11

TABLE 12. Reductions in anthracnose development in mangoes and avocadoes after post harvest treatment with SDBS. Fruit green (few, small or no lesions) or in semi-ripe (many lesions) condition.

Variety	No. of fruit in treatment	No. of days in storage	Disease Rating[1] of Controls	Disease Rating[1] of SDBS fruit
Common mangoes (few lesions)	5	7	3 ± 1	2 ± 1
Common mangoes (many lesions)	5	7	4 ± 0	3 ± 1
Common mangoes (3 treatments on consecutive days)	3	6	3 ± 1	1 ± 0
Common mangoes	3	6	3 ± 1	1 ± 1
Kensington mangoes[3]	10	6	2 ± 1	1 ± 1
Sensation mangoes[3] (small lesions)	10	8	3 ± 1	2 ± 1
Sensation mangoes[3] (no lesions)	10	8	4 ± 0	2 ± 1
Fuerte avocadoes[2]	10	10	60%	10%

1 Disease rating scale for mangoes: 1 = no infection to ¼ of surface with lesions, 2 = ¼ to ½ of surface with lesions, 3 = ½ to ¾ of surface withlesions, 4 = ¾ to whole of surface with lesions

2 Disease rating for avocadoes expressed as a percentage of fruit with lesions
3 fruit fly infestation

LEGENDS

TABLE 12

1 Disease rating scale for mangoes: 1 = no infection to ¼ of surface with lesions, 2 = ¼ to ½ of surface with lesions, 3 = ½ to ¾ of surface withlesions, 4 = ¾ to whole of surface with lesions

2 Disease rating for avocadoes expressed as a percentage of fruit with lesions

3 Fruit fly infestation

FIGURE 1

The effects of SDBS post harvest dip on the development of anthracnose on naturally infected mangoes after 7 days incubation at room temperature.

(a) Natural infection mild

(b) Natural infection severe

FIGURE 2

The effect of 3 dips and sticker-spreader on the development of anthracnose on naturally infected common mangoes.

FIGURE 3

Diagrammatic representation of fermentation process.

FIGURE 4

pH and organic acid profiles of the standard fermenation at 30°C (Mean of 3 replicate fermentations, Run 2N).

FIGURE 5

Increase in mean diameter of anthracnose lesions on naturally infected mango fruit over a 17 day period after treatment (mean 20 lesions on each of 15 fruit per treatment).

CLAIMS

1. A biocontrol agent which includes or is derived from a sourdough starter formulation comprising:

(i) a mixed culture of the yeast component and a bacterial component; and

(ii) a substrate for the mixed culture (i).

2. A biocontrol agent as claimed in Claim 1 wherein the yeast component comprises yeasts of the genus *Saccharomyces*.

3. A biocontrol agent as claimed in Claim 1 wherein the bacterial component comprises Lactobacilli.

4. A biocontrol agent as claimed in Claim 2 wherein the yeast component comprises *Saccharomyces cerevisiae*.

5. A biocontrol agent as claimed in Claim 3 wherein the Lactobacilli component comprises *L. parabuchneri*, *L. parabuchneri/brevis* strain and *L. casei ssp casei*.

6. A biocontrol agent as claimed in Claim 3 wherein *Lactobacilli* component comprises *L. buchneri*, *L. casei* and *L. brevis*.

7. A biocontrol agent as claimed in Claim 1 having been deposited at the Australian Government Analytical Laboratories on March 5, 1993 and having been allocated Accession No. N93/9578.

8. A process for reducing or alleviating fungal infection of plants or fruit including the step of application of a biocontrol agent as claimed in Claim 1 to such plants or fruit.

9. A process as claimed in Claim 8 wherein the starter sourdough formulation is mixed with cereals or molasses and also mixed with water at a pH in the range of 3.0-6.0 and thereafter allowed to ferment to form a fermentation broth which was then applied to plants or fruit infected with a fungus.

10. A process as claimed in Claim 9 wherein the fungus comprises *Colletrotrichum gloeosporioides* or *Colletotrichum acutatum*.

11. A process as claimed in Claim 9 wherein the fermentation broth is applied to mangoes or avocado.

12. A process for production of a biocontrol agent for use in biocontrol of fungal diseases in fruit or plants including the steps of:

(i) obtaining a sourdough starter culture including a yeast component and a bacterial component;

(ii) adding said starter culture to a fermentation substrate to form a fermentation broth;

(iii) incubating said fermentation broth for a period of time which is desirably 12-48 hours; and

(iv) forming a liquid from said fermentation broth into a biocontrol formulation and optionally adding a suitable carrier which may include or is selected from a spreader or sticker, dispersant, thickener, emulsifier or stabiliser.

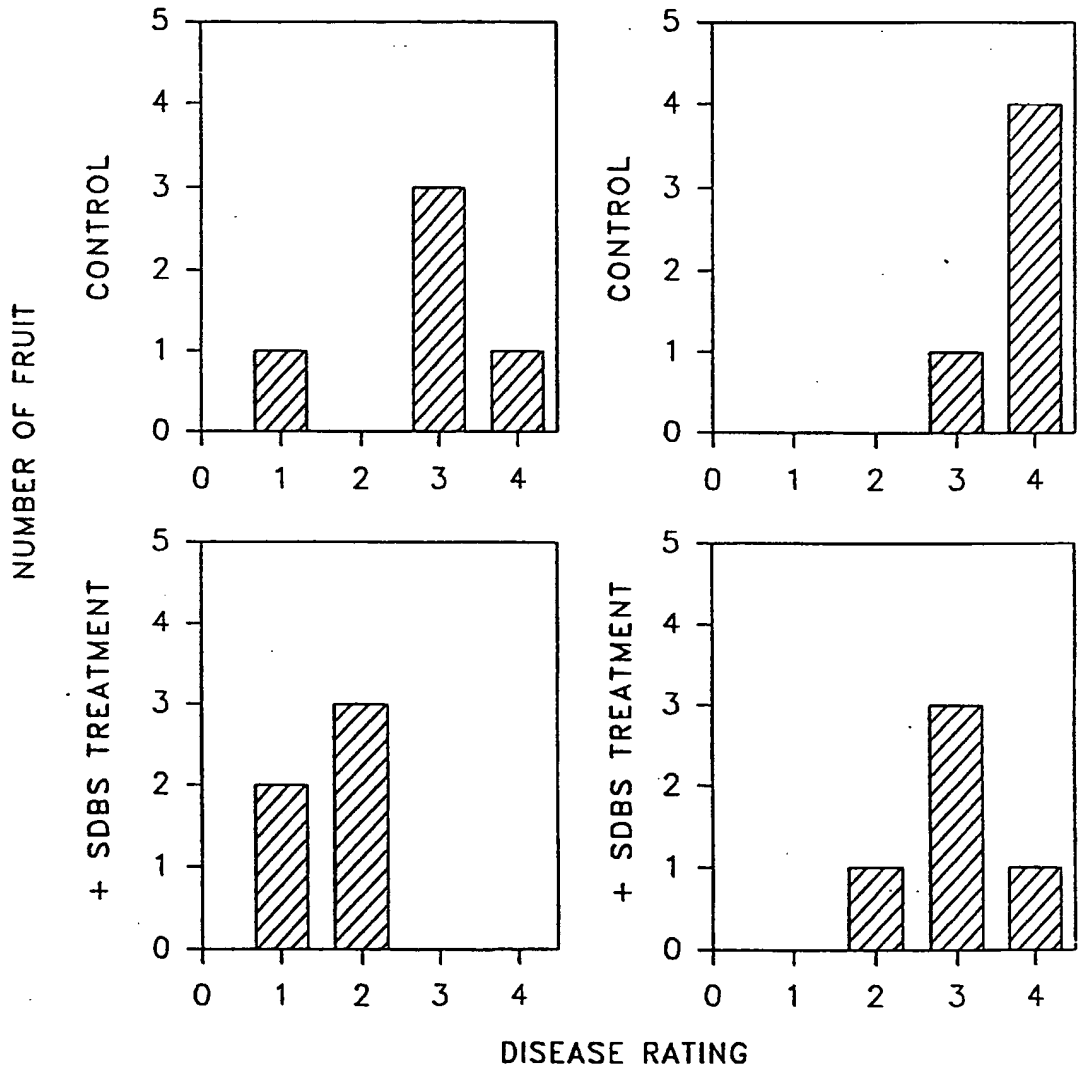
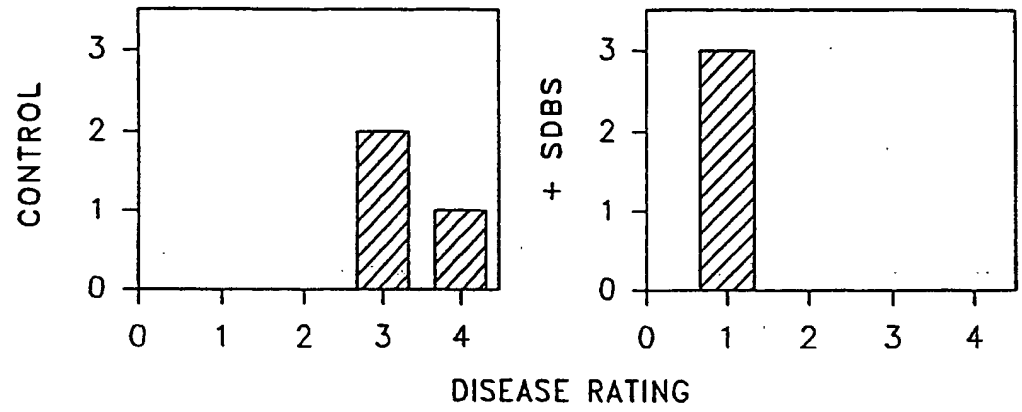

NUMBER OF FRUIT
CONTROL
+ SDBS TREATMENT
CONTROL
+ SDBS TREATMENT
0
1
2
3
4
5
DISEASE RATING
a) Natural infection mild
b) Natural infection severe

FIG. 1

a) The effect of dipping on days 1, 2 and 3 on the performance of SDBS



b) The effect of Nufilm 17 and Codacide on SDBS performance.

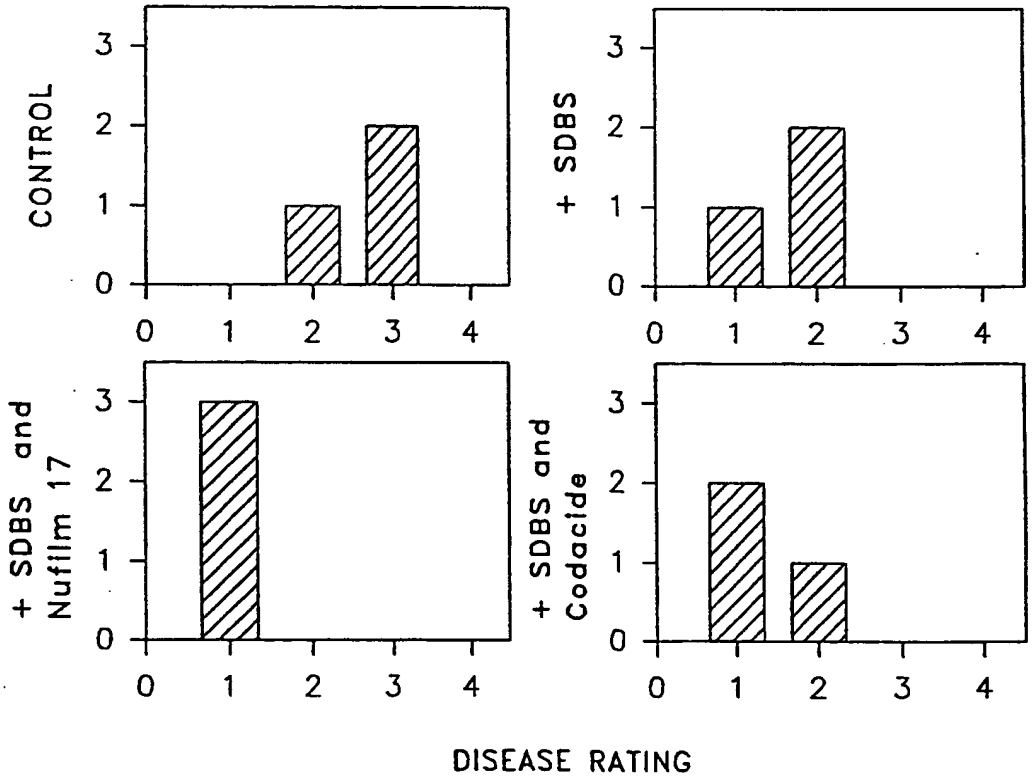


FIG. 2

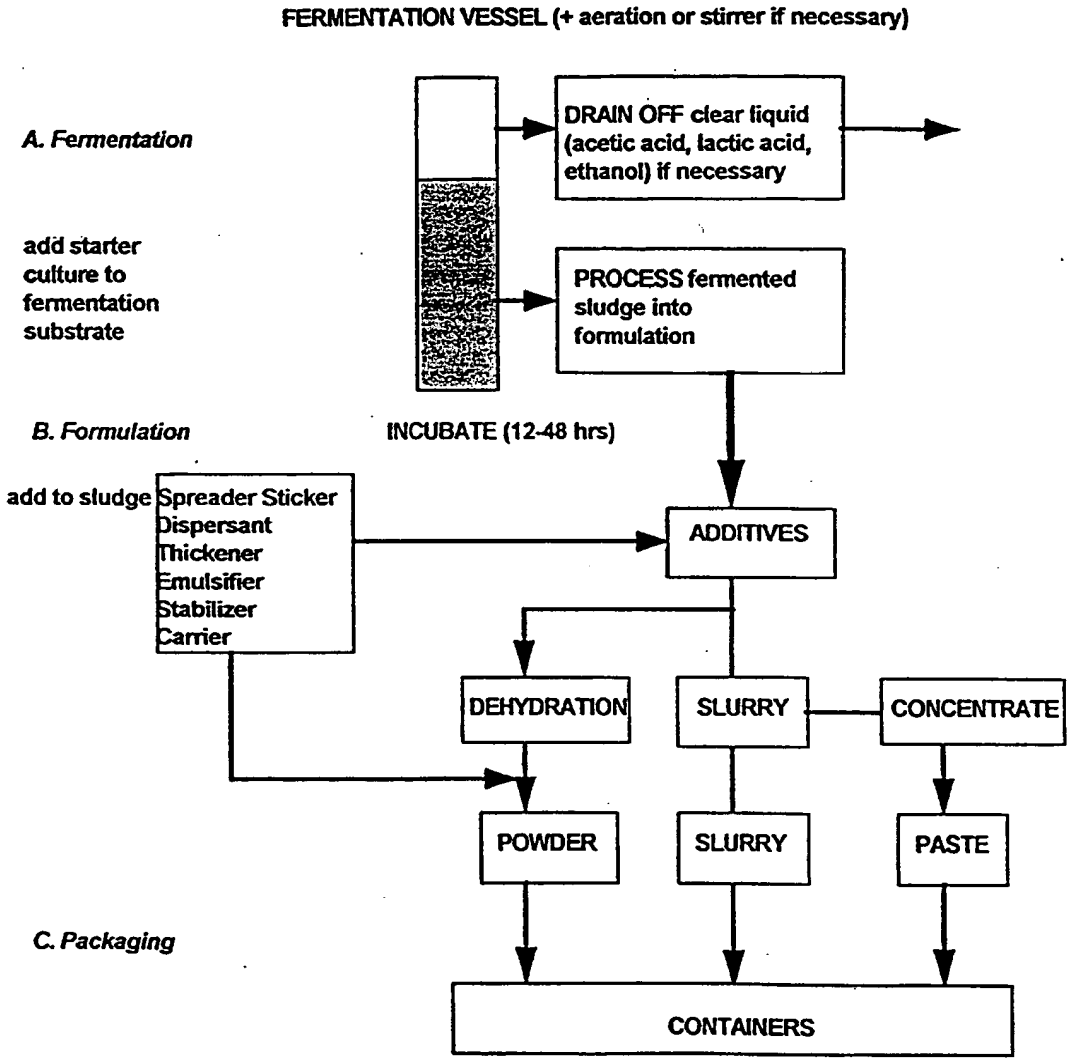

FERMENTATION VESSEL (+ aeration or stirrer if necessary)
A. Fermentation
DRAIN OFF clear liquid (acetic acid, lactic acid, ethanol) if necessary
add starter culture to fermentation substrate
PROCESS fermented sludge into formulation
B. Formulation
INCUBATE (12-48 hrs)
add to sludge
Spreader Sticker
Dispersant
Thickener
Emulsifier
Stabilizer
Carrier
ADDITIVES
DEHYDRATION
SLURRY
CONCENTRATE
POWDER
SLURRY
PASTE
C. Packaging
CONTAINERS

FIG. 3

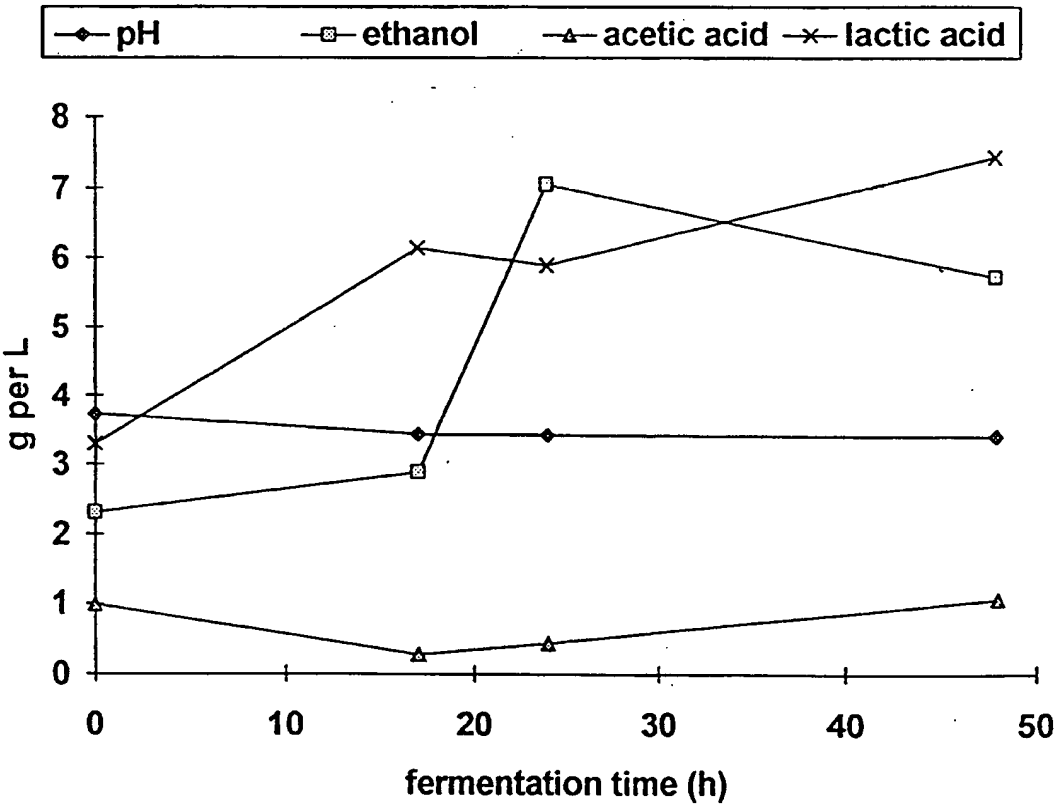

pH
ethanol
acetic acid
lactic acid
g per L
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
fermentation time (h)

FIG. 4

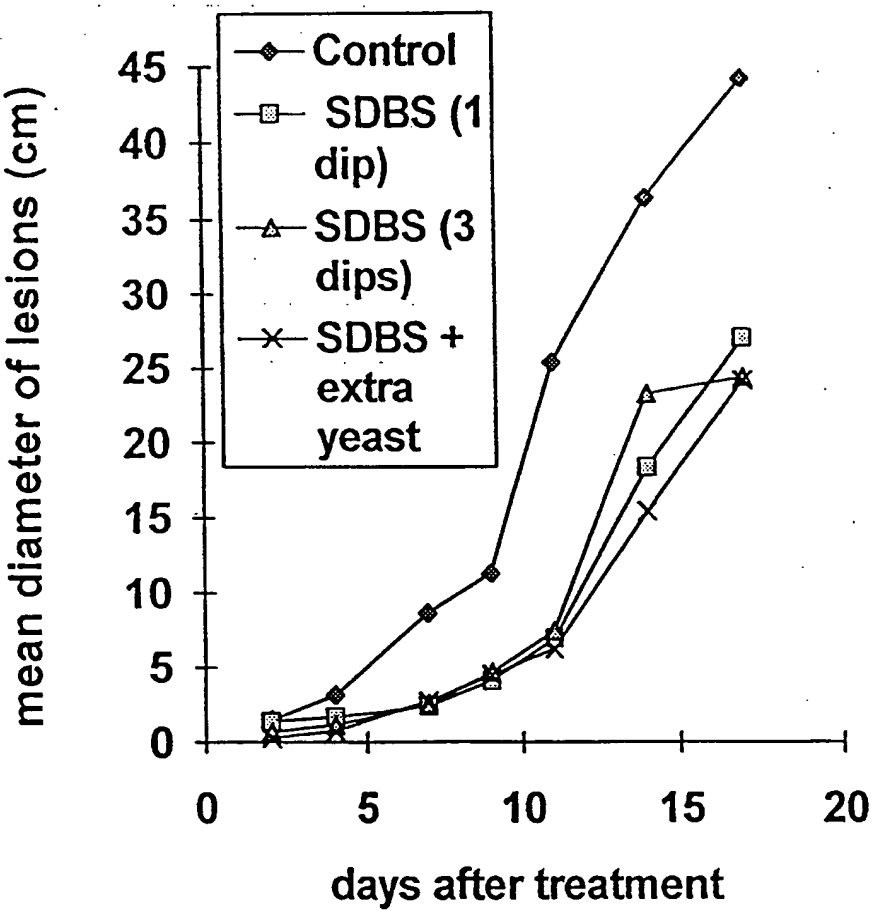

Control
SDBS (1 dip)
SDBS (3 dips)
SDBS + extra yeast
mean diameter of lesions (cm)
0
5
10
15
20
25
30
35
40
45
0
5
10
15
20
days after treatment

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 94/00110

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[5] A01N 63/02, 63/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A01N 15/00, 63/02, 63/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU:IPC as above

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT: YEAST OR SACCHROMYC: OR TORULOPS: SOURDOUGH: LACTOBAC
CAPRI: YEAST OR SACCHROMYC: OR TORULOPS: SOURDOUGH: LACTOBAC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Derwent WPAT Online Abstract Accession No. 90-335331, BR 8900938 (FIOCRUZ FUND O CRUZ) 9 October 1990 (09.10.90) Abstract	1-12
A	EP,A,517378 (MYCOGEN CORPORATION) 12 September 1992 (12.09.92) page 2, lines 51-57; page 4, lines 28-34; Claims 1 to 10	1-12
A	WO,A,92/18009 (DARATECH PTY. LTD.) 29 October 1992 (29.10.92) Abstract and Claims 1 to 49	1-12

continued....

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 1994 (15.06.94)	24 June 1994 (24.06.94)

Name and mailing address of the ISA/AU

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
A	AU,A,90558/91 (CROP GENETICS INTERNATIONAL CORPORATION) 29 May 1992 (29.05.92) Abstract and Claims 1 to 31	1-12
A	Derwent WPAT Online Abstract Accession No 88-261551, JP 63190808 (KOHRAN SANGYO KK) 8 August 1988 (08.08.88) Abstract	1-12
A	Derwent WPAT Online Abstract Accession No 88-253363, JP 63183506 (KYOWA HAKKO KOGYO KK) 28 July 1988 (28.07.88) Abstract	1-12
A	Derwent WPAT Online Abstract Accession No 87-279828, FR 2594294 (BREVIER C) 21 August 1987 (21.08.87) Abstract	1-12
P,A	Derwent WPAT Online Abstract Accession No 93-11926, JP 5168456 (HIGA T) 2 July 1993 (02.07.93) Abstract	1-12
P,A	Derwent WPAT Online Abstract Accession No 93-11925, JP 5168455 (HIGA T) 2 July 1993 (02.07.93) Abstract	1-12
A	Derwent WPAT Online Abstract Accession No 88-08939, JP 63107908 (FUMAKILA) 12 May 1988 (12.05.88) Abstract	1-12
A	Patent Abstracts of Japan, C-731, page 13, JP,A,2-91010 (TAKESHI MIURA) 30 March 1990 (30.03.90) Abstract	1-12
A	Patent Abstracts of Japan, C-3, page 110, JP,A,55-9022 (SUMITOMO KAGAKU KOGYO KK) 22 January 1980 (22.01.80) Abstract	1-12
A	Patent Abstract of Japan, C-51, page 11, JP,A,56-8682 (HIROTO NAKADA) 29 January 1981 (29.01.81) Abstract	1-12

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member		
BR,A, 8900938			
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JP 63183506			
FR 2594294			
JP 5168456			
JP 5168455			
JP 63107908	JP,A, 63107908 JP,A, 62269086 KR,B, 9108504 US,A, 4877667	CN,A, 1040283 CN,A,87105175 WO,A,8800755 CN,B, 1014562	JP,A, 62272817 CN,B, 1007187 EP,A, 277235 JP,A, 62054040
JP 2-91010			
JP 55-9022			
JP 56-8682			

END OF ANNEX